XCVIII. THE CALCIFICATION *IN VITRO* OF KIDNEY, LUNG AND AORTA.

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ALTHOUGH calcification in the animal body is normally confined to hypertrophic cartilage and osteoid, in certain abnormal or pathological conditions deposits of calcium salts may also be formed in other tissues, among which are the kidney, the lung and the arteries. Some of these conditions, for example hypervitaminosis D, are associated with exceptionally high concentrations of blood-calcium or phosphate, suggesting that the plasma has become by some agency supersaturated with calcium salts. In others degenerative changes in the tissues themselves precede, and may possibly be the primary cause of, calcification.

Without attempting to reproduce *in vitro* all the abnormal conditions concerned it seemed of importance to discover whether the calcification of such tissues could be induced in our experimental solutions, and if so, in what respects the process would differ from that taking place in hypertrophic cartilage and osteoid. Experiments of this kind, though carried out with the tissues of normal animals, might yet throw light on abnormal calcification and, by differentiation, on the normal process of calcification in the skeleton.

Two questions of particular interest were: (*a*) whether the phosphatase which occurs in some of these tissues is at all responsible for their calcification, and (*b*) whether these tissues possess in any degree properties akin to those which constitute the second mechanism of cartilage.

EXPERIMENTAL.

The technique was similar to that employed for experiments on the calcification *in vitro* of cartilage and bone. Calcifying solutions of similar composition were used [Robison and Rosenheim, 1934]. They are referred to as solution 8 : 6 : 10, 8 : 6 : 0, *etc.*, according to their content of calcium, inorganic P and organic P respectively in mg. per 100 ml. of basal salt solution. The organic phosphorus was in the form of α-glycerophosphoric ester.

Our experiments were confined to the kidney, lung and aorta. The tissues were obtained from normal rats and rabbits of various ages and in a few cases from young rachitic rats. They were excised as rapidly as possible, with precautions to avoid bacterial contamination, and small pieces were immersed in the warm calcifying solutions at p_H 7·4 and left at 37° for various periods. Subsequently these pieces were fixed in 10 % neutral formalin, washed, dehydrated and embedded in paraffin. Serial sections were cut, stained with silver nitrate and counterstained. A black deposit of metallic silver formed in the

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sections on exposure to light was accepted as evidence of the presence of solid phosphate or carbonato-phosphate of calcium, that is, of tissue calcification. The deposits obtained were never so heavy as to be readily apparent before sectioning and staining.

Pieces of each tissue, fixed without immersion in the calcifying solutions, were sectioned and stained as controls.

In some experiments small pieces of the same kidney, lung or aorta were left for different lengths of time in samples of the same calcifying solution, each piece in a separate flask. In other experiments the pieces of each tissue were immersed for the same length of time but in a number of solutions differing in their content of calcium, inorganic phosphate and phosphoric ester.

The results obtained show that calcification of kidney, lung and aorta *in vitro* can be realised under suitable experimental conditions, but that such calcification takes place much more slowly than that of hypertrophic cartilage and is much more erratic in occurrence and distribution.

No attempt has been made to group the results of individual experiments together, since these were often irregular, one piece of tissue possibly showing calcification in 4 days while another piece of the same tissue might fail to calcify in a longer period or in a solution of higher Ca × P level.

The positive and negative results obtained with rat's kidney and lung are collected in Table I and those obtained with the aortae of rats and rabbits in Table II.

From Table I it will be seen that calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but that the deposits were not generally formed in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and not once in lung, although a large number of experiments were performed with highly supersaturated inorganic solutions and the period of immersion was extended to 4 days.

Table I. *Calcification of kidney and lung* in vitro.

		Inorganic solutions		Solutions containing phosphoric ester			
mg. per 100 ml. Ca		8	8	8	8	8	8
mg. per 100 ml. P inorg.		5	6	4	5	6	4
mg. per 100 ml. P org.		0	0	10	10	10	30
Tissue	Days						
Kidney	1	– – – –	– – – –	– –	– –	– – –	+
		.	– – – –	.	.	– – –	– –
	2	– –	+	+	–	+	+ +
		.	– –	.	.	.	.
	3	–	–	.	.	.	.
	4	–	–	.	.	.	.
Lung	1	– –	– –	.	–	–	–
	2	–	–	.	–	+ +	+
	3	.	.	.	.	.	+
	4	–	–	.	+	+	.

Each sign in Tables I and II refers to a separate piece of tissue, calcification *in vitro* being indicated by + and absence of calcification by –.

In the aortae both of rats and rabbits, however, calcification did occur in the inorganic solutions, although not invariably and never in less than 3 days (Table II). These solutions were very highly supersaturated and would certainly have produced regular and extensive calcification in cartilage within 10–16 hours.

Table II. *Calcification of aorta* in vitro.

		Inorganic solutions			Solutions containing phosphoric ester			
mg. per 100 ml.	Ca	8	8	8	8	8	8	8
	P inorg.	4	5	6	4	5	6	4
	P org.	0	0	0	10	10	10	30
Species	Days							
Young rat	1	.	−	.	−	.	.	−
	2	.	− −	−	−	.	−	−
	3	.	− −	−	−	.	−	−
	4	.	−	− −	− −	− −	−	.
	5	.	−	− −	−	−	+	.
		.	.	.	.	.	−	.
Full-grown rat	1	.	−	−	−	−	−	.
	2	.	−	.	−	−	−	.
	3	.	−	+	−	.	−	.
	4	.	+	− − −	−	+ + +	+	−
		.	−	.	.	− − −	−	.
	5	.	+	+ +	.	+	+ +	.
		.	.	− − −	.	−	− − −	.
	6	.	−	− −	.	+	+	.
		.	.	.	.	.	− −	.
	7	.	.	+	.	−	.	.
	10	.	.	.	.	.	+	.
Young rabbit	3	.	−	.	.	−	.	.
	4	−	−	.	.	.	.	.
	5	− −	+ +	+	.	− −	−	.
		.	−	.	.	.	.	.
	6	.	+	.	.	−	.	.
Full-grown rabbit	2	.	.	− − (− −)	.	.	−	.
	3	+	− −	− −	.	.	−	.
	4	.	.	+	.	.	.	.
	5	+	+ (+ +)	+	.	−	−	−
		.	− − (− −)	−	.	.	.	.
	6	.	.	+	.	.	.	.
	8	.	+	+	.	.	−	.

Signs in brackets refer to lengths of aorta treated with acetone or desiccated (see text).

In rat's aorta calcification was somewhat more frequent and was denser in presence of phosphoric ester but no such effect was observed in rabbit's aorta. This result was expected, since it has been shown that the former tissue contains phosphatase [Macfarlane *et al.*, 1934], while the latter is devoid of the enzyme [Kay, 1928]. From the results quoted the presence of glycerophosphoric ester would indeed appear to have had some inhibitory effect on the calcification of rabbit's aorta, and this too is reasonable, since the unhydrolysed ester must compete with the inorganic phosphate for calcium ions.

It was noted that the deposits formed in the aorta whether in presence or absence of ester became more extensive and denser as the period of immersion was prolonged and also that the aortae of full-grown rats and rabbits became calcified more readily than those of younger animals. On the other hand, there was no evidence that the age or condition of the animal influenced the calcification of kidney and lung *in vitro*. These tissues were taken from young rachitic rats, young normal rats and adult rats, and positive and negative results were distributed fairly evenly among the three groups.

Effect of acetone and of desiccation on the calcifiability of the rabbit's aorta.

It has previously been shown that the second calcifying mechanism of hypertrophic cartilage is seriously damaged by treatment of the cartilage with acetone or alcohol, or by desiccation [Robison *et al.*, 1930]. Experiments were, therefore, performed to discover whether similar treatment would affect the calcifiability of rabbit's aorta which possesses no phosphatase. The aortae of three full-grown rabbits were used. One portion of each aorta was immersed directly in the calcifying solution while another portion was first soaked in acetone for 22 hours and a third dried over sulphuric acid *in vacuo*. In two experiments solution 8 : 5 : 0 was used and the period of immersion was 5 days. In the third experiment the solution was 8 : 6 : 0 and the period only 2 days. Calcification was obtained in one of the 5-day experiments in the two lengths of aorta which had been treated with acetone and desiccated respectively (Plate III, Fig. 4), but not in the control piece. There was no calcification in any part of the aortae used for the other two experiments. It is clear, therefore, that the calcifiability of the aorta is not diminished but rather enhanced by acetone treatment and desiccation, and that the qualities leading to the calcification of this tissue are markedly different in this respect from the second mechanism of hypertrophic cartilage.

Description of the deposits.

(*a*) *Kidney.* Calcification *in vitro* was usually confined to a narrow zone of cortex beginning a few cell layers from the exterior, though in some cases the extreme peripheral zone was the most densely calcified. The deposits were finely granular and extended over the basement membranes of the tubules and often throughout the tubule cells. The nuclei of these cells also appeared to be calcified (Plate III, Fig. 1). Deposits were frequently observed in the glomeruli and on the glomerular capsules. The distribution of the deposits formed in the rat's kidney *in vitro* bears some resemblance to that of the calcification in the same tissue described by Brand and Holtz [1929] and produced by overdosage with irradiated ergosterol.

Although half kidneys or transverse slices were used in these experiments so that at least one cut surface of the medulla was always exposed to the solution, only slight traces of calcification were observed in this region. In this connection it is of interest that the cortex has been shown by Kay [1926] to possess considerably greater phosphatase activity than the medulla.

(*b*) *Lung.* The deposits obtained in lung were not localised in any special part of the organ. There was diffuse granular deposition, denser in some places than in others and extending irregularly throughout the sections examined. It was not confined to the cells lining the alveoli but appeared also in the alveolar spaces. There was fairly heavy calcification in the walls of the bronchioles (Plate III, Fig. 2).

(*c*) *Aorta.* Calcification *in vitro* in different regions of the same aorta was very varied in amount, being usually densest in the ascending aorta and aortic arch (Plate III, Fig. 3). Its distribution thus corresponded with that of the calcified areas observed in the aorta in experimental hypervitaminosis D in the rat and the rabbit as described by Duguid [1930] and Vanderveer [1931] respectively.

Calcification commenced most often on the outer side of the media or in the adjacent layers of the adventitia; sometimes, however, it occurred in the middle or inner layer of the media, while the outer layers remained free from deposit. Occasionally the calcified zone extended in places almost through the entire

thickness of the vessel wall though adjoining parts of the wall might be free from deposit (Plate IV, Fig. 8).

The elastic fibres in the broad medial coat are the most prominent feature seen in the stained transverse sections of the aortic arch and dorsal aorta of rats and rabbits. The intima is not well developed. The bands of deposit stretched along and between the elastic laminae (Plate IV, Figs. 5 and 7) and the fine granules of calcium salt very often appeared to have been formed on the fibres themselves (Plate IV, Fig. 8). Relatively large rounded aggregates definitely located at intervals along the fibres were sometimes seen (Plate IV, Fig. 6). Wenzel [1928] has observed the deposition of calcium salts in granular form on the elastic fibres in experimental hypervitaminosis D; while deposits in the media, between the elastic laminae, have been described by Kreitmair and Hintzelmann [1928].

Various experiments on calcification of the aorta.

Certain further experiments which were essentially of a preliminary nature may be briefly referred to here.

1. Although there was little evidence of marked degeneration in the tissues except after the most prolonged periods of immersion, less obvious physico-chemical changes must have been taking place from the time of excision. That such degenerative changes might be essential for calcification seemed the more possible in view of the relative slowness with which calcification set in and its sporadic appearance.

In one of the experiments undertaken to investigate this point, an excised rat aorta was kept 3 days at 37° in a solution of Ca × P product too low to produce calcification. Pieces of the tissue were then transferred to the more concentrated solutions 8 : 6 : 0 and 8 : 5 : 10 and left for further periods of 1, 2 or 4 days. Calcification did not occur in less than 4 days after transference of the tissues to the latter solutions. Such degeneration of the tissue as may have occurred during the preliminary period did not, therefore, serve to produce more rapid calcification than usual.

In other experiments sections of aorta were cut on the freezing microtome and alternate sections were stained for fat and calcium salts. Although the stainable fat was found to increase in amount with increasing periods of immersion at 37° there seemed to be no connection between the occurrence and location of fatty degeneration and the occurrence of calcification.

2. With the usual technique the pieces of aorta immersed in the experimental solutions remained in a highly contracted condition very different from that of the aorta *in vivo*. In a few experiments an attempt was made to maintain part of the vessel wall in a distended state. A piece of the aorta was slit open and stretched between the ground ends of two lengths of glass tubing, which were clamped together. A few ml. of 8 : 6 : 10 solution were forced through the tissue drop by drop at a pressure of about 160 mm. Hg over a period of 4 days. During this time solution and tissue were maintained at 37°. Calcification, however, did not take place.

3. Calcification of certain tissues is known to occur as a result of injury. A length of aorta was, therefore, purposely injured by scraping with a sterile scalpel or by pinching with forceps before immersion in the calcifying solutions. Such injury did not lead to increased calcification *in vitro*.

4. Since permeability of the aorta wall may be a factor limiting the rate of calcification and since testicular extract has been shown to exert a marked effect in increasing tissue permeability [McClean, 1931], an experiment was

carried out in which 0·2 ml. of this extract (for which we are indebted to Drs McClean and Morgan) was injected into the media of a rabbit's aorta before excision. There was no evidence of calcification in this aorta after 3 days in 8 : 5 : 0 solution.

Discussion.

If the results of these experiments are to be applied to the problems of pathological calcification it is clear that great caution must be used. In the body deposits of calcium salts are frequently found in abnormal or necrotic tissues whereas the calcification which was obtained *in vitro* occurred in tissues taken for the most part from normal animals. The deposits obtained in the arteries *in vitro* would, indeed, appear to bear less resemblance to those occurring in such conditions as Mönckeberg's sclerosis or atheroma (associated with muscular and fatty degeneration respectively) than to those formed in the early stages of experimental hypervitaminosis D in which tissue degeneration is relatively slight and appears to be of secondary nature [Wenzel, 1928; Vanderveer, 1931]. In the lung and kidney also the calcification *in vitro* was not obviously related to any marked degenerative changes; though doubtless some tissue degeneration must have occurred during the prolonged period of immersion.

There are grounds for assuming that in hypervitaminosis D the condition of supersaturation of the circulating fluid with calcium salts is a determining factor in tissue calcification, as it is in our experimental solutions. In necrotic or degenerate tissues factors of a different type are introduced.

In connection with the etiology of arterial disease it is of some interest that in our experimental solutions calcification occurred in isolated pieces of aorta subjected to no mechanical stresses and not distended by the force of the blood stream.

The experiments described in this paper have shown that in a medium supersaturated with calcium salts, calcification of certain tissues other than hypertrophic cartilage and osteoid may occur without participation of phosphatase, although if the tissue contains this enzyme and phosphoric ester is present the likelihood of deposition will be increased. These results do not suggest that the tissue phosphatases are essential factors in pathological calcification[1]. At least one of the tissues whose calcification was studied, the aorta, possesses qualities which can provoke deposition of calcium salts in the tissue from supersaturated solutions before spontaneous precipitation occurs in the solution itself. By this empirical criterion we should recognise that the aorta possesses the second mechanism, but the slow and erratic formation of the deposits indicates a very low degree of activity. It is not proved that the mechanism in the aorta is identical with that in cartilage. There is, indeed, some evidence to the contrary, for example, in the different effect of acetone on these two tissues. In the aorta the qualities which favour calcification are perhaps associated with the surface of the fibres. It is not unlikely that similar forces at fibrillar interfaces may also assist deposition in cartilage, even if the high activity of the second mechanism in this tissue should ultimately prove to be due to an enzyme complex, as we have suggested elsewhere [Robison and Rosenheim, 1934].

The results of these experiments again emphasise the very special position occupied by hypertrophic cartilage and osteoid with respect to the dual calcifying mechanism. They show also that certain other tissues possess qualities

[1] In heterotopic ossification, in which true osteoid tissue is developed in abnormal locations, phosphatase probably plays the same part as in normal bone formation [Huggins, 1931].

which, although unable to effect their calcification under normal conditions, may do so if the calcium-phosphate level in the blood is abnormally raised. Whether such qualities become further developed as a result of tissue degeneration must be left for future investigation.

SUMMARY.

1. Calcification of the kidney, lung and aorta excised from normal animals has been realised *in vitro* by immersion of the tissues in experimental solutions of similar composition to those used for calcification *in vitro* of hypertrophic cartilage.

2. Calcification of these tissues took place much more slowly than that of hypertrophic cartilage, and especially in the aorta, was erratic in occurrence and distribution.

3. Calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but not usually in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and never in lung.

4. Calcification occurred, though not invariably, in aortae both of rats and rabbits immersed in very highly supersaturated inorganic solutions for periods of 3 to 8 days.

5. The deposits formed in the kidney, lung and aorta *in vitro* had some resemblance in appearance and distribution to those described as occurring in the same tissues *in vivo* in experimental hypervitaminosis D.

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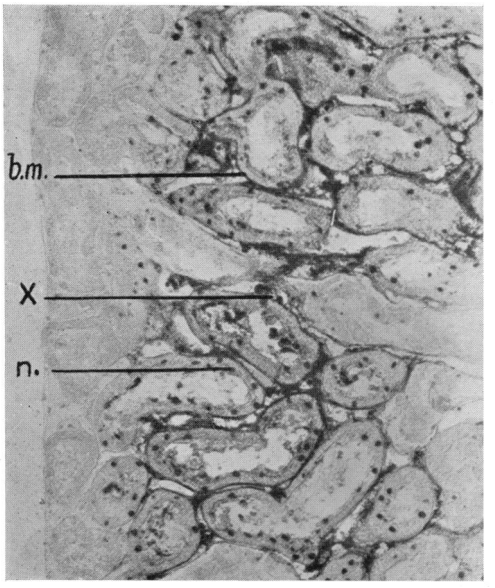


Fig. 1.

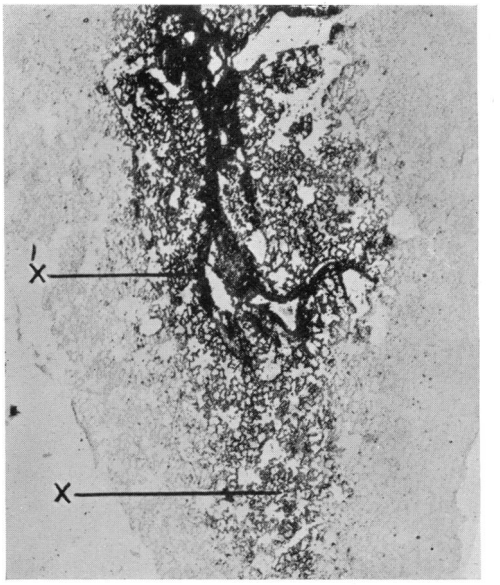


Fig. 2.

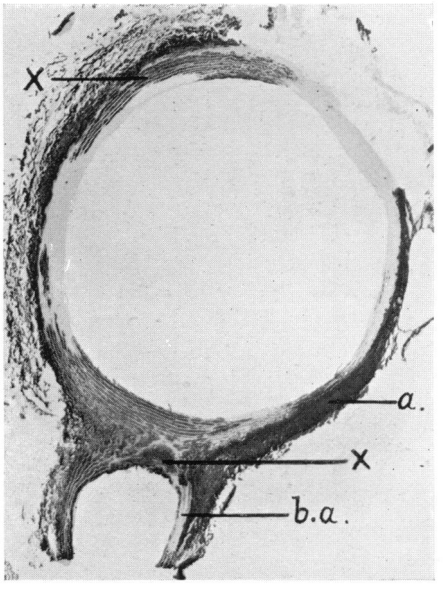


Fig. 3.

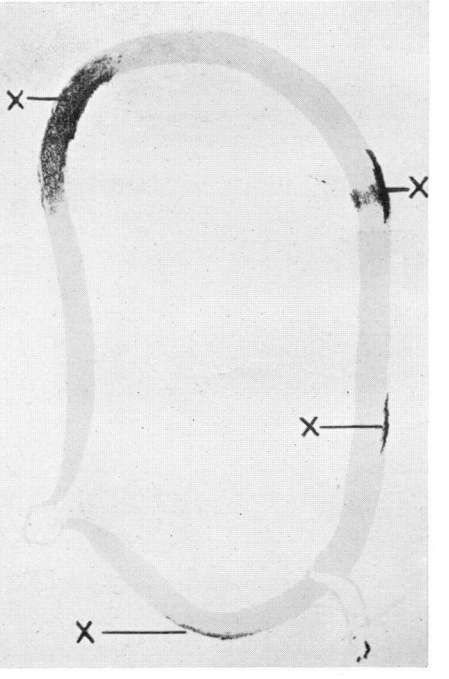


Fig. 4.

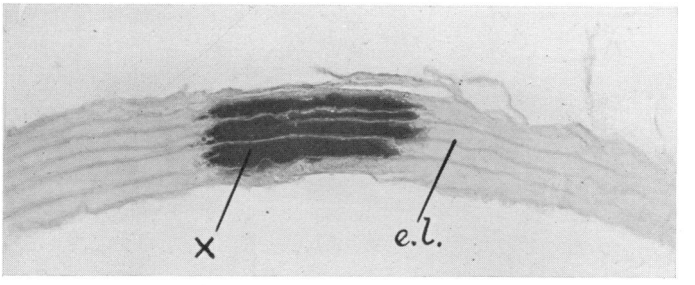
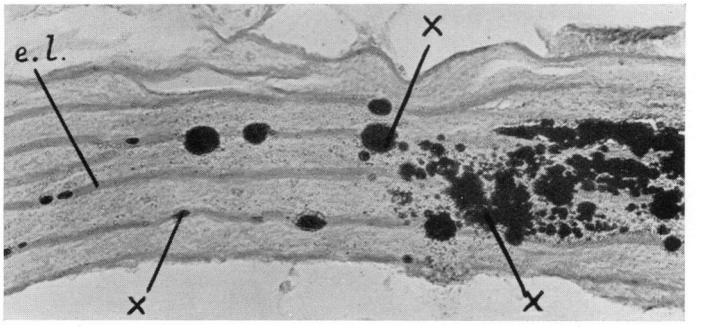


Fig. 5.



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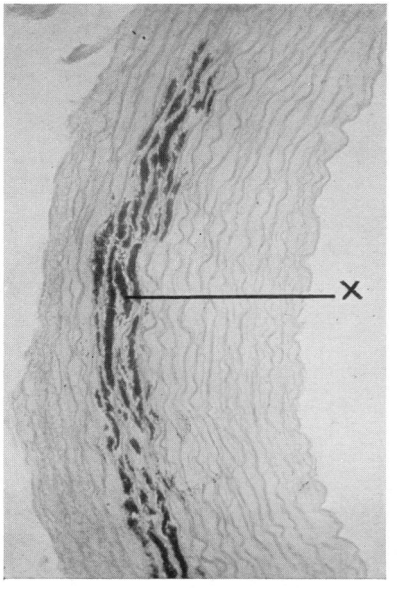

Fig. 7.

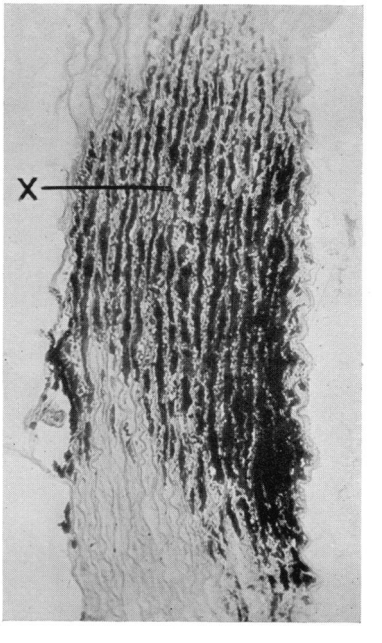

Fig. 8.

DESCRIPTION OF FIGURES IN PLATES III AND IV.

Sections of kidney, lung and aorta, cut after immersion of the tissues in various calcifying solutions for the periods specified. The sections were treated with silver nitrate to show the tissue calcification (*X*) which occurred *in vitro*.

a. = aorta.
b.a. = branch artery.
b.m. = basement membrane of tubule cell.
e.l. = elastic lamina.
n. = nucleus of tubule cell.

PLATE III.

Fig. 1. Cortex of the kidney of a full-grown rat. 2 days in solution 8 : 4 : 30. Note the calcification in the basement membrane of the tubules and in the nuclei of the tubule cells. (Mag. × 250.)

Fig. 2. Lung of the same rat. 4 days in solution 8 : 5 : 10. (Mag. × 27.)

Fig. 3. Transverse section of the aortic arch of a full-grown rat. 4 days in solution 8 : 5 : 10. Note the heavy calcification of the adventitia and media in the main and branch arteries. (Mag. × 22.)

Fig. 4. Aorta of rabbit. The tissue was soaked for 22 hours in acetone and subsequently immersed for 5 days in solution 8 : 5 : 0. The section illustrates the patchy distribution of calcification *in vitro* noted also in tissues not subjected to acetone treatment. (Mag. × 20.)

PLATE IV.

Fig. 5. Thoracic aorta of a full-grown rat. 3 days in solution 8 : 6 : 0. Note the deposit apparently located in the bands of muscle and connective tissue between the elastic laminae. (Mag. × 140.)

Fig. 6. Part of the same aorta as in Fig. 3. 5 days in solution 8 : 6 : 0. Note the rounded aggregates apparently formed in and on the elastic laminae. (Mag. × 350.)

Fig. 7. Aorta of full-grown rabbit. 3 days in solution 8 : 4 : 0. Slight calcification was obtained in the outer half of the media. The deposit is apparently located between the elastic laminae. (Mag. × 140.)

Fig. 8. Aorta of full-grown rabbit. 5 days in solution 8 : 5 : 0. Note the heavy calcification extending almost through the entire thickness of the artery wall. The deposit is chiefly between the laminae, but where it is densest it would seem to overlap them. In places fine granules are visible on the fibres between the bands of deposit. (Mag. × 140.)